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## Overproduction, crystallization and preliminary X-ray characterization of Abn2, an endo-1,5- $\alpha$ -arabinanase from *Bacillus subtilis*

Two *Bacillus subtilis* extracellular endo-1,5- $\alpha$ -L-arabinanases, AbnA and Abn2, belonging to glycoside hydrolase family 43 have been identified. The recently characterized Abn2 protein hydrolyzes arabinan and has low identity to other reported 1,5- $\alpha$ -L-arabinanases. Abn2 and its selenomethionine (SeMet) derivative have been purified and crystallized. Crystals appeared in two different space groups: *P*1, with unit-cell parameters  $a = 51.9$ ,  $b = 57.6$ ,  $c = 86.2$  Å,  $\alpha = 82.3$ ,  $\beta = 87.9$ ,  $\gamma = 63.6^\circ$ , and *P*<sub>2</sub><sub>1</sub><sub>2</sub><sub>1</sub>, with unit-cell parameters  $a = 57.9$ ,  $b = 163.3$ ,  $c = 202.0$  Å. X-ray data have been collected for the native and the SeMet derivative to 1.9 and 2.7 Å resolution, respectively. An initial model of Abn2 is being built in the SeMet-phased map.

### 1. Introduction

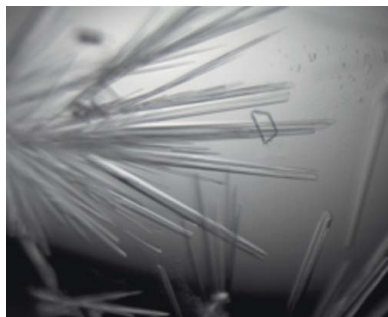
Arabinan is an L-arabinose homoglycan present in plant tissues that is generally associated with pectins. This polysaccharide is composed of  $\alpha$ -1,5-linked L-arabinofuranosyl units, some of which are substituted with  $\alpha$ -1,3- and  $\alpha$ -1,2-linked chains of L-arabinofuranosyl residues (Beldman *et al.*, 1997). The two major enzyme families that hydrolyse arabinan are  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55; AFs) and endo-1,5- $\alpha$ -L-arabinanases (EC 3.2.1.99; ABNs). AFs remove arabinose side chains, allowing ABNs to attack the glycosidic bonds of the arabinan backbone, releasing a mixture of arabino-oligosaccharides and L-arabinose as the products of the reaction. Arabinanases have several applications, in particular in the food industry, such as food technology and nutritional medical research (Beldman *et al.*, 1997).

*Bacillus subtilis*, a Gram-positive endospore-forming bacterium which participates in plant-biomass degradation, synthesizes two extracellular ABNs belonging to glycoside hydrolase family 43 (GH43; Coutinho & Henrissat, 1999), AbnA and Abn2 (YxiA), which hydrolyze sugar-beet arabinan (branched), linear  $\alpha$ -1,5-L-arabinan and pectin (Inácio & de Sá-Nogueira, 2008; Leal & de Sá-Nogueira, 2004). The three-dimensional structures of three different 1,5- $\alpha$ -L-arabinanases, Arb43A from *Cellvibro japonicus* (Nurizzo *et al.*, 2002), BsArb43A (AbnA) from *B. subtilis* (Proctor *et al.*, 2005) and ABN-TS from *B. thermodenitrificans* (Yamaguchi *et al.*, 2005), have been determined by X-ray crystallography. These enzymes, which vary in length from 323 to 347 amino acids, display a unique motif consisting of a five-bladed  $\beta$ -propeller fold. Since the recently characterized Abn2 from *B. subtilis* (Inácio & de Sá-Nogueira, 2008) shows less than 23% amino-acid identity to these 1,5- $\alpha$ -L-arabinanases and is a much larger enzyme (469 residues) than other related arabinanases, the determination of its three-dimensional structure should provide valuable information concerning the structural features of glycoside hydrolase family 43 arabinanases. Here, we report the crystallization and preliminary X-ray analysis of the GH family 43 Abn2 from *B. subtilis*.

### 2. Experimental procedures

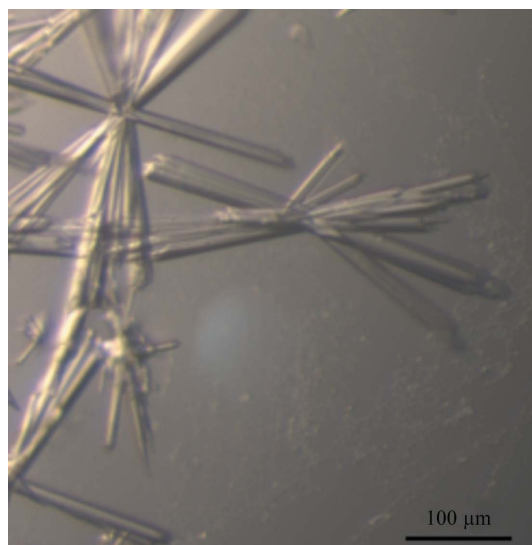
#### 2.1. Overproduction and purification

The overproduction and purification of Abn2 in *Escherichia coli* BL21 (DE3) pLysS has been described previously (Inácio & de Sá-Nogueira, 2008). Briefly, the *abn2* allele (GenBank accession No.

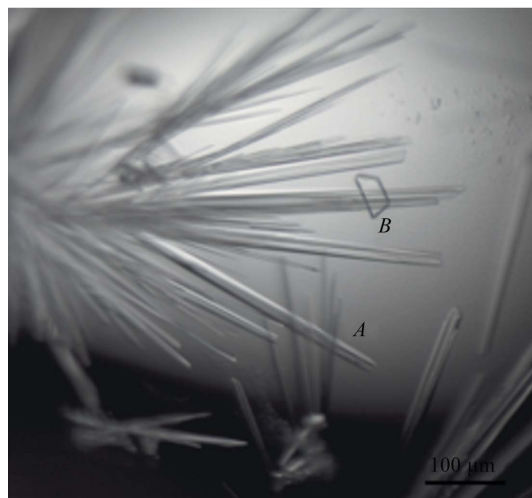


EU373814) was amplified by PCR with primers ARA237 (5'-GGC-GAATTGTTCAATATGTTCAACCG-3') and ARA238 (5'-CGCTTCTCCCTCGAGTTTAGATCCC-3') using chromosomal DNA of wild-type strain *B. subtilis* 168 T<sup>+</sup> as template. The resulting 1568 bp DNA fragment was digested with *NdeI*-*XhoI* unique restriction sites introduced by the primers (bold sequence) and cloned into the same sites of pET30a(+) (Novagen). In the resulting plasmid pZI39, the wild-type primary sequence of the Abn2 is fused directly to the carboxyl-terminal sequence (LEHHHHHH) encoded by pET30a(+).

*E. coli* BL21 (DE3) pLysS cells harbouring pZI39 were grown at 310 K and 160 rev min<sup>-1</sup> in 1 l LB with an appropriate antibiotic selection. When the OD<sub>600</sub> reached 0.6, expression of Abn2 was induced by the addition of 1 mM IPTG. The culture was grown for an additional 4 h at 310 K and 160 rev min<sup>-1</sup>. Cells were harvested by centrifugation at 277 K and 8000g for 10 min. All subsequent steps were carried out at 277 K. The periplasmic protein fraction (PPF) was prepared by osmotic shock and loaded onto a 1 ml HisTrap column (Amersham Pharmacia Biotech). The Ni-bound proteins were eluted



(a)



(b)

**Figure 1**

(a) SeMet-derivative crystals of Abn2. The dimensions of a typical crystal fragment used in data collection were  $0.05 \times 0.05 \times 0.2$  mm. (b) Crystals of the native Abn2 crystals. Two crystalline forms are present. Crystal form A belongs to space group *P1* and has typical dimensions of  $0.02 \times 0.06 \times 0.1$  mm; crystal form B belongs to space group *P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>*.

**Table 1**

Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	SeMet	Wild type
Wavelength (Å)	0.97865	1.03320
Space group	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	<i>P1</i>
Unit-cell parameters (Å, °)	<i>a</i> = 57.9, <i>b</i> = 163.3, <i>c</i> = 202.0	<i>a</i> = 51.9, <i>b</i> = 57.6, <i>c</i> = 86.2, $\alpha$ = 82.3, $\beta$ = 87.9, $\gamma$ = 63.6
Resolution limits (Å)	127.0–2.70 (2.85–2.70)	85.4–1.90 (2.00–1.90)
$R_{\text{merge}}^{\dagger}$	0.093 (0.190)	0.047 (0.136)
$R_{\text{p.i.m.}}^{\ddagger}$	0.056 (0.121)	0.045 (0.036)
Total No. of reflections	365808 (46766)	134405 (17357)
No. of unique reflections	53601 (7661)	67116 (8980)
Mean $I/\sigma(I)$	17.6 (7.4)	14.5 (6.4)
Completeness (%)	99.9 (99.6)	95.7 (87.1)
Multiplicity	6.8 (6.1)	2.0 (1.9)
$R_{\text{ano}}^{\S}$	0.064 (0.112)	
Anomalous completeness (%)	99.1 (95.0)	
Anomalous multiplicity	3.5 (3.2)	

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$ ,  $\ddagger R_{\text{p.i.m.}} = \frac{\sum_{hkl} [N/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$ ,  $\S R_{\text{ano}} = \frac{\sum_h |I(h) - \langle I(-h) \rangle|}{\sum_h [I(h) + \langle I(-h) \rangle]}$ , where  $\langle I(h) \rangle$  indicates the mean intensity of symmetry-related reflections of the same Bijvoet mates *h*.

with a discontinuous imidazole gradient and fractions containing Abn2 that were more than 95% pure were dialyzed overnight against a buffer containing 100 mM Tris pH 8.0, 100 mM NaCl, 10% (v/v) glycerol. Purified protein was then concentrated using an Amicon Ultra 15 Centrifugal filter (Millipore) to a final concentration of 10.9 mg ml<sup>-1</sup> and stored in the same buffer at 193 K. Seleno-L-methionine-labelled protein was overexpressed in an auxotrophic *E. coli* strain B834 (DE3) harbouring pZI39. A colony was used to inoculate 10 ml LB with an appropriate antibiotic selection and the culture was incubated for 6 h at 150 rev min<sup>-1</sup> and 310 K. The culture was then diluted in a 1:20 ratio with 100 ml of SeMet Base media (Molecular Dimensions Ltd), L-methionine, Nutrient Mix (Molecular Dimensions Ltd) and an appropriate antibiotic selection. After overnight incubation the cell pellet was separated by centrifugation at 3000g and 298 K for 10 min, washed to remove residual L-methionine and resuspended in 1 l of the same medium containing L-selenomethionine (SeMet; Molecular Dimensions Ltd) instead of L-methionine. When the OD<sub>600</sub> reached 0.75, induction was performed by the addition of 1 mM IPTG. The culture was grown for an additional 3 h and cells were then harvested. Preparation of the PPF and purification of SeMet-Abn2 was carried out as described above for the native protein. Purified protein was concentrated to a final concentration of 15.4 mg ml<sup>-1</sup> using an Amicon Ultra 15 Centrifugal filter (Millipore) and stored in the same buffer as the native protein at 193 K.

## 2.2. Crystallization

Preliminary crystallization screens were carried out with the native protein using a sitting-drop vapour-diffusion experimental setup (100 nl protein solution added to 100 nl reservoir solution equilibrated against 100  $\mu$ l reservoir solution) using a Cartesian Nanodrop Robot to set up 96-well plates at 294 K. Crystals were found in condition No. 22 [0.1 M HEPES pH 7.5, 70% (v/v) MPD] of Classics Screen (Nextal). Crystal growth was scaled up by hanging-drop vapour diffusion at 294 K in 48-well plates (1  $\mu$ l protein solution and 1  $\mu$ l reservoir solution equilibrated against 200  $\mu$ l reservoir solution), obtaining the best shaped crystals in 65% (v/v) MPD, 0.1 M Tris pH 8.5. The crystals that appeared showed two distinct morphologies (Fig. 1). Final crystal dimensions were  $40 \times 50 \times 200$   $\mu$ m for crystal form A and  $20 \times 60 \times 100$   $\mu$ m for crystal form B. The SeMet-protein

crystallization experiment was set up using the same conditions as used for the native protein, but crystals appeared only in one crystalline form (Fig. 1). The best-shaped crystals were obtained with 62% (v/v) MPD and 0.1 M Tris pH 8.4.

### 2.3. X-ray analysis

Crystals were transferred into a stabilizing solution containing 70% (v/v) MPD and 0.1 M Tris pH 8.5 and subsequently flash-cooled in liquid nitrogen. Native protein crystals with different morphologies belonged to different space groups: crystal form *A* belonged to space group  $P2_12_12_1$ , with unit-cell parameters  $a = 57.81$ ,  $b = 164.48$ ,  $c = 203.31$  Å, and can accommodate four molecules in the asymmetric unit with a Matthews coefficient  $V_M = 2.29$  Å<sup>3</sup> Da<sup>-1</sup> and a solvent content of 46%, while crystal form *B* belonged to space group  $P1$ , with unit-cell parameters  $a = 51.97$ ,  $b = 57.64$ ,  $c = 86.27$  Å,  $\alpha = 82.31$ ,  $\beta = 87.97$ ,  $\gamma = 63.66^\circ$ , which is compatible with the presence of two molecules in the asymmetric unit, corresponding to a Matthews coefficient  $V_M = 2.17$  Å<sup>3</sup> Da<sup>-1</sup> and a solvent content of 43% (Matthews, 1985). As the  $P1$  crystals were the better diffracting crystal form, a data set was collected from these native crystals to 1.9 Å resolution at ID29, ESRF (Grenoble) at 100 K in 180 non-overlapping 1° oscillation images with a crystal-to-detector distance of 265.56 mm. Data were indexed with *MOSFLM* (Leslie, 2006) and scaled with *SCALA* (Evans, 2006).

The SeMet derivative of Abn2 only crystallized in one crystal form, which was similar to form *A* of the wild-type protein. SeMet-derivative crystals diffracted to 2.7 Å resolution and belonged to space group  $P2_12_12_1$ , with unit-cell parameters  $a = 57.84$ ,  $b = 163.22$ ,  $c = 201.85$  Å, which can accommodate four molecules (each containing 13 selenomethionine residues) in the asymmetric unit, corresponding to a Matthews coefficient of 2.26 Å<sup>3</sup> Da<sup>-1</sup> and a solvent content of 46%.

SAD data were collected from an SeMet-labelled crystal at 100 K at the BM14 Station at ESRF at a wavelength of 0.97865 Å, which was chosen after an absorption scan measurement to maximize the anomalous signal of Se atoms. Oscillation steps of 0.2° over a range of 180° were applied with a crystal-to-detector distance of 291.89 mm. SeMet-derivative crystal diffraction images were indexed with *MOSFLM* (Leslie, 2006) and scaled with *SCALA* (Evans, 2006).

### 2.4. Structure solution

The program *SOLVE* (Terwilliger & Berendzen, 1999) was used to locate 31 of the 42 Se atoms in the asymmetric unit and to perform initial phasing to a figure of merit of 33% (*Z* score 54.3). Subsequently, *RESOLVE* (Terwilliger, 2000) was used to perform statistical density modification with noncrystallographic symmetry averaging to a final figure of merit of 72%. In the final map, autotracing using *RESOLVE* (Terwilliger, 2003) succeeded in building some main features of the four independent molecules in the asymmetric unit, resulting in the placement of 765 residues out of 1876. Manual model building with *Coot* (Emsley & Cowtan, 2004) is in progress.

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